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FABIO DE CARVALHO PEIXOTO

EVALUATION OF THE ABILITY OF MILTEFOSINE ASSOCIATED WITH TOPIC GM-CSF IN MODULATE THE IMMUNE RESPONSE OF PATIENTS WITH CUTANEOUS LEISHMANIASIS.

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Trabalho de Conclusão de Curso apresentado à Escola Bahiana de Medicina e Saúde Pública, como parte dos requisitos para obtenção do título de Bacharel em Biomedicina.

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Esta monografia foi julgada adequada à obtenção do grau de Bacharel em Biomedicina e aprovada em sua forma final pelo Curso de Biomedicina da Escola Bahiana de Medicina e Saúde Pública.

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EVALUATION OF THE ABILITY OF MILTEFOSINE ASSOCIATED WITH TOPIC GM-CSF IN MODULATE THE IMMUNE RESPONSE OF PATIENTS WITH CUTANEOUS LEISHMANIASIS.

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Abstract

Cutaneous leishmaniasis (CL) due to *L. braziliensis* is associated with an exaggerated inflammatory response and tissue damage. Miltefosine is more effective than antimony (Sb^v) in the treatment of CL and here we evaluate the ability of miltefosine and GM-CSF to modify the immune response in participants of a clinical trial aimed to compare the efficacy of miltefosine *plus* GM-CSF vs miltefosine *plus* placebo vs antimony in CL caused by *L. braziliensis*. Patients were allocated in three groups of treatment. Miltefosine *plus* GM-CSF, Miltefosine *plus* placebo and Sb^v. Mononuclear cells were obtained from those patients on day 0 and day 15 of therapy and cultured with or without soluble *leishmania* antigen for 72 hours. Granzyme-B, IFN-y, TNF and IL-1β, were determined in supernatants by ELISA. The lymphocyte proliferation was evaluated, utilizing Ki-67 as marker, by flow cytometry. The oxidative burst was evaluated in presence of *L. braziliensis* by flow cytometry, with dihydrorhodmine as marker, and monocytes were cultured with *L. braziliensis* (5:1) for 2, 48 and 72 hours for evaluation of infection ratio through optical microscopy. We observed that patients treated with miltefosine *plus* GM-CSF have decreased levels of Granzyme B, but increased levels of IL-1β during treatment compared to before therapy and higher production of IFN-γ and TNF than Sb^v treated ones. There was an increase in the proliferation of $CD4^+$ T cells in patients using miltefosine and in $CD8^+$ T cells when GM-CSF was associated, and an increase in the oxidative burst after *L. braziliensis* infection in miltefosine *plus* GM-CSF group on day 15 of therapy. Moreover, the number of *L. braziliensis* in infected monocytes as well as the percentage of infected was higher after 2 hours and lower after 48 and 72 hours in cells from patients treated with miltefosine *plus* GM-CSF. In patients treated only with

miltefosine the same effects were observed after 2 and 72 hours. In this study we show that in addition to the ability of miltefosine to kill *leishmania*, the modulation of the immune response caused by miltefosine and GM-CSF may increase the cure rate of CL patients using these drugs.

1 Introduction

Cutaneous leishmaniasis (CL) in Latin America is predominantly caused by *Leishmania (Viannia) braziliensis* and is characterized by the presence of one or a few well delimitated ulcerated lesions with granulomatous fundus and elevated borders (1). Host immunological factors play an important role in the pathogenesis of the disease. Mononuclear cells from patients with CL stimulated with soluble *leishmania* antigen (SLA) displays an exacerbated Th1 type immune response and produce high levels of IFN-γ, TNF and low levels of IL-10 in cultures (2). The production of IFN- γ and TNF are important to prevent parasite proliferation in mononuclear phagocytes and the dissemination of the infection (3, 4). However, this response is not capable to eliminate all the parasites and the persistent stimulation of the immune system by parasites and *Leishmania* antigens lead to an exaggerated inflammatory response resulting in tissue damage (5). Furthermore, studies have shown a pathogenic role of $CD8^+$ T cells at the lesion site, as the lyses of infected cells release molecules that induce the secretion of IL-1β, TNF, inflammasome activation and the appearance of the ulcer (6). In this context, inflamatory cytokines have an important role in parasite eradication, but its over production is associated with tissue damage and development of the cutaneous ulcer.

Meglumine antimoniate (Sb^v) is the first-choice drug for treatment of CL in Latin America, but an increase in therapeutical failure, hitting over 50% of the patients has been observed in the last 15 years (7,8). Miltefosine is an oral leishmanicidal drug that acts blocking cytochrome C oxidase leading to changes in mitochondrial membrane potential (9) and its able to eliminate up to 95% of *L. donovani* and *L. infantum* amastigotes in mice (10). Miltefosine has been effective in the treatment of CL caused by *L. braziliensis* and *L. guyanensis* (7, 11). In Brazil miltefosine cured 75% of patients infected with *L. braziliensis* and 71.4% of patients infected with *L. guyanensis* (7,11).

The host immune response has a great impact and influence on therapeutic response of CL. Patients with diffuse CL, a disease caused by *L. amazonensis* in Latin America, have a poor Th1 type immune response and are refractory to therapy (12). Nevertheless, the presence of the immune response as observed in CL patients infected with *L. braziliensis* does not induce a fast healing of the disease. Different from the majority of infectious diseases where early therapy is associated with fast healing, the failure rate of Sb^v therapy in patients in the pre ulcerative phase of the disease, called early CL, is over than 70% and do not prevent the appearance of the ulcer (13,14). Previous studies showed that Sb^v associated to granulocyte and macrophage colony stimulation factor (GM-CSF) or pentoxifylline (drug that decreases TNF production) are more effective and reduce healing time of cutaneous and mucosal leishmaniasis (15,16,17,18). The GM-CSF induces *in vitro* macrophage activation and increase leishmania killing (19,20,21). The miltefosine has not only the ability to kill *Leishmania* but also enhances chemotaxis, motility, monocyte adhesion and phagocytosis (22). Studies indicate that the ability of miltefosine in stimulate macrophage and monocyte activation is due to its semblance with phosphatidylcholine, enhancing the membrane fluidness of this cells (22). The aim of this study is to determine if miltefosine applied by oral route and topical GM-CSF may modify the immune response of CL patients treated with these drugs.

2 Article types

The article type is Original Research (https://www.frontiersin.org/journals/immunology#article-types). The author guideline can be found at: Author Guidelines.

3 Materials and Methods

3.1 Patients

Participants of this study were patients with CL from the endemic area of Corte de Pedra, Bahia, Brazil who were participating of an ongoing randomized control study aimed to compare the efficacy and effectivity of Miltefosine associated to the GM-CSF versus Miltefosine *plus* placebo and antimoniate of meglumine. The diagnosis of CL was by the presence of a typical CL ulcer and detection of DNA of *L. braziliensis* by PCR. Patients were allocated in three groups: 1, Miltefosine (2,5mg/Kg/d for 28 days with maximum dose of 150mg/day orally) + topic GM-CSF (gel cream 0,01%, twice a day per 28 days); 2, Miltefosine in the same dose and schedule of group 1 + topic placebo (gel cream twice a day per 28 days) and group 3 that received Sb^v (20mg/Kg/d intravenously per 20 days in the maximum dose of 1200mg). Blood of these individuals were collected before treatment (day 0) and during therapy on the day 15. Inclusion criteria were age between 18 and 60 years old, illness duration more than 20 and less than 90 days and size of the ulcer between 10 and 40 mm.

3.2 Ethical Statement

All patients agreed to participate of the study and signed an informant consent. This study was approved by the Institution Review Board of the Federal University of Bahia Medical School and the National Commission of Ethics in Research (CONEP).

3.3 Immunological Studies:

3.3.1 Separation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood by density gradient centrifugation using Ficoll-paque (GE Healthcare). Cells were washed twice in saline and were resuspended at the desired concentration in RPMI 1640 (GIBCO BRL., Grand Island, NY USA) supplemented with 10% of BFS (GIBCO BRL., Grand Island, NY USA) and antibiotics.

3.3.2 Infection with *L. braziliensis*

To evaluate the infection rate 2,5 x 10^6 cells/mL were putted in Nunc® Labtek® plates, after 2 hours, necessary time for monocyte adhesion, the non-adherent cells were washed out of the plate. Once monocytes were isolated, they were infected with *L. braziliensis* (5 parasites per monocyte) for two hours then the remaining promastigotes were washed out the plate. Afterwards the cells were cultured for 2, 48 or 72 hours at 37º C with 5% CO_2, the supernatants were collected for cytokine quantification (IL-1β, TNF, IL-6 e IL-10). The slides were stained with panoptic for later quantification of infected monocytes and the number of amastigotes per 100 monocytes, which was done through optic microscopy.

3.3.3 Oxidative burst quantification

To evaluate the reactive oxygen species, 1 x 10^6 PBMC were treated with dihydrorhodamine-123 at 10ng/mL (Cayman Chemical Company) for 10 minutes. After that, cells were infected with *Leishmania braziliensis* (Lb), 5 *leishmania* per monocyte, considering that this cell type is 15% of total PBMC, for 25 minutes and then marked with αHLA-DR, αCD14. The fluorescence intensity of the cells was evaluated by flow cytometer and data were analyzed through FlowJo®.

3.3.4 Lymphocyte proliferation essay

To evaluate the lymphocyte proliferation, 1 x 10^6 PBMC were cultured in presence or absence of SLA (5µg/mL). After 5 days of incubation at 37º C with 5% CO_2, cells were marked with conjugated antibodies αCD4 and αCD8, with the goal of separate the lymphocyte subpopulations, and αKi-67 as cell proliferation marker. Afterwards cells were evaluated by flow cytometer and data were analyzed by Flowjo®.

3.3.5 Cytokine production determination

The PBMC were adjusted to 3 x 10^6/mL in complete RPMI and cultured in a 37º C, CO_2 incubator for 72 hours in presence or absence of SLA (5 µg/mL). The supernatants of those cultures were collected and utilized for measurement of Granzyme B, IL-1β, IL-10, IFN-γ, TNF, and the chemokines CXCL9 and CXCL10, through ELISA sandwich as previously described.

3.4 Statistical analysis

Statistical analysis was performed using the Wilcoxon test for paired variables and Mann-Whitney rank test for unpaired measurements *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All experiments were statically analyzed through Prism GraphPad® 8.0.2, such as the graphics elaboration.

4 Results

4.1 Infection ratio

Monocytes were isolated through adhesion in Nunc® labtek® chambers, infected with *L. braziliensis* and cultured for 2, 48 and 72 hours. After 2 hours of incubation we could observe that monocytes from miltefosine *plus* GM-CSF and miltefosine *plus* placebo treated patients had higher infection ratio on day 15 of therapy than before treatment, 49(45-53) *versus* 40(39-43) (p=.0079) and 47(43-50) *versus* 42(40-45) (p=.02) (figure 1a), as well as a higher number of amastigotes internalized per 100 monocytes, 266(205-277) *versus* 181(169-201) (p=.008) and 203(196-222) *versus* 169 (155-197) (p=.015) (figure 1d).When monocytes were obtained on day 15 and were cultured for 48 hours there was a decrease in the infection ratio in miltefosine *plus* GM-CSF group, 49(44-53) *versus* 56(55-60) at day 0(p=.03) (figure 1b), and a decrease after 72 hours by both miltefosine *plus* GM-CSF, 39(36/40) in day 15 *versus* 45(43/49) in day 0 (p=.008), and miltefosine *plus* placebo (p=.009), treated groups was observed(figure 1c). The same phenomena were observed when comparing the number of amastigotes during vers*us* before therapy. At day 15 of therapy when monocytes were cultured for 48 hours there was a decrease in parasites internalized by monocytes form miltefosine *plus* GM-CSF group (p=.03) (figure 1e) and after 72 hours miltefosine *plus* GM-CSF treated group decreased from 153(139-162) to 114(90-133) (p=.008) and the same was

observed in patients treated with miltefosine *plus* placebo decreased (p=.008) (figure 1f). When comparing groups, we observed higher percentage of infected cells in miltefosine *plus* GM-CSF group compared to antimony, 49(45-53) *versus* 40(37-47) (p=.02) (figure 1a) and a higher number of amastigotes internalized by monocytes from the first group of patients 266(205-277) compared to miltefosine *plus* placebo (p=.03) and Sb^v 176(166-193) (p=.007) group after 2 hours of incubation (figure 1d). Miltefosine *plus* placebo group also presents higher levels of amastigotes internalization than meglumine antimoniate treated patients (p=.008) (figure 1d)., After 48 hours the relation between miltefosine *plus* GM-CSF and antimony groups inverts, and patients from the second group presents a higher percentage of infected cells (p=.016) (figure 1b) as well as a higher number of amastigotes per 100 monocytes (p=.015) (figure 1e). Analyzing 72 hours cultures, we saw lower infection ratio in both miltefosine *plus* GM-CSF, 39(36-40), and miltefosine *plus* placebo, 39(35-42), groups compared to antimony treated patients, 43(42-46) (p=.007) (p=.02) (figure 1c), as well as lower number of amastigotes internalized 114(90-133) and 124(105-146) *versus* 160(140-171) (p=.007)(p=.016)(figure 1f).

4.2 Oxidative burst

The reactive oxygen species (ROS) produced by monocytes from CL patients after 25 minutes of infection with *L. braziliensis* before and on day 15 of therapy is shown in figure 4. The median fluorescence index (MFI) of DHR 123 of monocytes from patients treated with miltefosine *plus* GM-CSF enhanced from 25(8-31) to 44,5 (19-81) from day 0 to day 15 (p=.03) (figure 2b). There was no difference in the oxidative burst during therapy in the other groups.

4.3 Lymphocyte proliferation

PBMC were cultured for 5 days for evaluation of $CD4^+$ and $CD8^+$ T cells proliferation through Ki-67 expression. To accomplish that, we quantified the frequency of T cells expressing this molecule in day 15 and divided by the frequency found in day 0, allowing the analysis of drug capacity in inducting proliferation. Using this index, we observe an increase in $CD4^+$ T proliferation in patients treated with miltefosine *plus* GM-CSF and miltefosine *plus* placebo, whereas a decrease in Sb^v group were noted. The same procedure was done with $CD8^+$ T cells and, with miltefosine *plus* GM-CSF as exception, a decrease in proliferation was observed during the treatment (figure 3b).

4.4 Cytokine production

PBMC from CL patients treated with miltefosine *plus* GM-CSF, miltefosine *plus* placebo or antimony were stimulated with SLA before and on day 15 of therapy and the production of Granzyme B, IFN-γ, TNF, IL-1β, IL-10, CXCL9 e CXCL10 were compared among the groups. The granzyme B production (figure 4a) in patients treated with miltefosine *plus* GM-CSF decreased from 2888pg/ml (1654-3111pg/ml) in day 0 to 1401 (436-2564pg/ml) in day 15, (p=.0001). The same effect was observed in miltefosine *plus* placebo treated patients in which granzyme B production decreased from 2574pg/ml (418-3219pg/ml) in day 0 to 1657 (650,2-1998pg/ml) on day 15 (p=.0021). When comparing groups among each other during treatment (day 15) we could observe lower levels of this cytokine in miltefosine *plus* GM-CSF group than in miltefosine *plus* placebo (p=.01) and meglumine antimoniate 2183pg/ml (1810-3102pg/ml) (p<.0001). Regarding IFN-γ (figure 4b), patients treated with miltefosine *plus* GM-CSF presented higher levels on day 15 of therapy, 1627pg/ml (162-6351pg/ml), than patients that received miltefosine *plus* placebo,

433,5pg/ml (0-9739pg/ml) (p=.048), or Sb^v 239pg/ml (0-742pg/ml), (p=.0032). The TNF levels (figure 4c) observed in patients insert in this same group of treatment (MF *plus* GM-CSF) were 654pg/ml (244-3127pg/ml) and in patients treated with miltefosine *plus* placebo were 879pg/ml (54-2500pg/ml) which were higher than those found in patients treated with meglumine antimoniate 382pg/ml (0-1550pg/ml), (p=.03)(p=.046). The IL-1β production (figure 4d) in patients treated with miltefosine *plus* GM-CSF increased from 38pg/ml (8-92pg/ml) on day 0 to 128pg/ml (26-300 pg/ml), (p=.01). Neither of other treatment options modified this cytokine production. Levels of CXCL10 (figure 4e) were higher in miltefosine *plus* GM-CSF, 1687pg/ml (1177-20000pg/ml) group than in Sb^v 1384,5pg/ml (15-2087pg/ml) (p=.03). Regarding IL-10 and CXCL9 no statistical difference has been observed.

5 Discussion

As tissue damage and ulcer development in American tegummentary leishmaniasis is mediated mainly by an exaggerated immune response, the use of immunomodulators in combination with leishmanicidal drugs is more effective than leishmanicidal drugs isolated, reduce the healing time and increase the cure ratio. Miltefosine is effective against visceral and cutaneous leishmaniasis and the cure rate of this drug is higher than that observed with meglumine antimoniate in ATL. However, miltefosine also has immunomodulatory properties. Miltefosine increases phagocytosis and enhances IFN-γ production, the main cytokine that activate macrophages for *Leishmania* killing. The GM-CSF has a wide effectivity on monocyte and macrophage activating these cells and granting leishmanicidal effect (19,20,21). Furthermore, in mice infected with *Mycobacterium tuberculosis*, this molecule inducts the recruitment of macrophages and lymphocytes to the site of lesion (23). We have previously showed that GM-CSF associated to meglumine antimoniate increases the cure rate and reduce the healing time of cutaneous leishmaniasis. In the present study taking advantage of an ongoing clinical trial evaluating the efficacy of miltefosine *plus* GM-CSF vs miltefosine *plus* placebo vs meglumine antimoniate, we compared the immunological response of CL patients before and during therapy. We observed that patients using miltefosine *plus* GM-CSF increased the respiratory burst and decreased the percentage of infected cells as well as the number of amastigotes per 100 monocytes during treatment. Moreover, there was an increase in the percentage of $CD4^+$ and $CD8^+$ T cells proliferation, an increase in IL-1β production and decrease of Granzyme B concentration.

Previous studies have shown that miltefosine changes the membrane fluidity of monocytes and macrophages, which might enhance phagocytic function by these cells (22). When those cells were stimulated *in vitro* with miltefosine and cultured with *Saccharomyces cerevisiae,* the drug enhanced phagocytosis by macrophages as well as the number of cells engaged in this activity (24). Those findings may support our results, as we found a high percentage of cells infected with *L. braziliensis* at early times with high amounts of amastigotes per monocyte in patients treated with miltefosine. After 48- and 72-hours culture, monocytes from patients treated with miltefosine showed lower frequency of infection when compared to the ones from patients treated with Sb^v and before treatment. We also observed an increase in the production of reactive oxygen species (ROS) by monocytes from patients insert in both miltefosine treated groups. Those data might be associated since previous studies have shown that ROS production is associated with parasite killing by monocytes (4).

Regarding cytokine production, we observed that miltefosine treatment keeps IFN-γ and TNF levels, while patients treated with Sb^v decrease the levels of those molecules during therapy. Previous studies show that IFN-γ and TNF are necessary to the control of parasite proliferation, granting leishmanicidal effects by mononuclear phagocytes (3,4), thus, it's

possible that, in addition of the leishmanicidal effect of miltefosine the maintenance of IFN-y and TNF levels in these groups may also contribute to parasite killing and with a higher cure rate in patients treated with miltefosine + GM-CSF and miltefosine + placebo than in those treated with meglumine antimoniate. We also observed an enhancement in IL-1β production as well as an increase in $CD4^+$ T cells, that could be associated with the destruction of a large number of parasites by miltefosine and the release of more antigen and consequently lymphocyte activation. We also observed that PBMC from patients insert in both miltefosine treated groups produced lower levels of granzyme B and perforin (data not shown) during treatment than before the beginning of therapy, which could also be associated with an increase in the cure rate, since those cytokines are associated with tissue damage and lyses of infected cells leading to inflammatory cytokines release.

Our results confirm that miltefosine enhances monocytic function and also show that this drug enhance IL-1B production and maintain the levels of IFN and TNF observed before therapy. We also showed that topical use of GM-CSF associated with oral miltefosine modify the systemic immune response increasing… This observation may contribute to a better parasite control and an increase in the cure rate of patients with cutaneous leishmaniasis treated with miltefosine.

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7 Author Contributions

FCP: Participated of all parts of the study with exception of patient's treatment. MTN and RSC: Assisted FCP to perform the experiments. JS: Diagnostic of the patients. LHG and PRM: Treatment of the CL patients. GP and MBN: Writing of the manuscript. LPC: Design of experiments, analysis of results and writing of the manuscript. EMC: Design of experiments, treatment of patients, analysis of results and writing of the manuscript.

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Figures

Figure 1

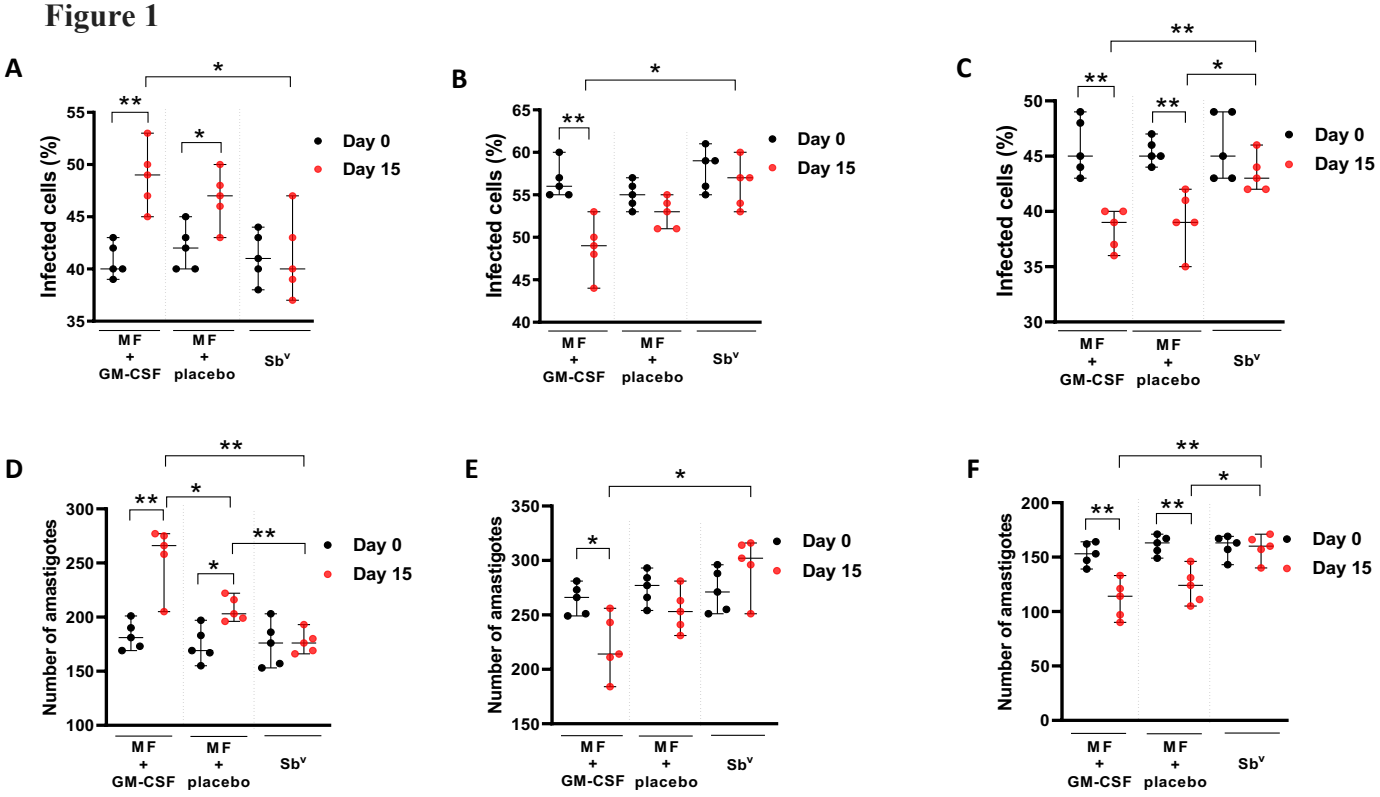

Fig 1. Influence of Miltefosine and GM-CSF treatment in the phagocytosis and killing of *L. braziliensis* by monocytes from CL patients. Monocytes from CL patients treated with Miltefosine + GM-CSF (n=5), miltefosine + placebo (n=5) and Sb^v (n=5) were infected with *L. braziliensis* promastigotes at a 5:1 ratio for 2, 48 and 72 hours. The percentage of infected cells after 2 hours (A), 48 hours (B) and 72 hours (C) as well as the number of intracellular parasites after 2 (D), 48 (E) and 72 (F) hours were determined by microscopic evaluation after panoptic staining on day 0 and day 15 of therapy. Statistical analyses were performed using the Mann-Whitney test for unpaired groups and Wilcoxon rank test for paired measurements *p<.05, **p<.01.

Figure 2

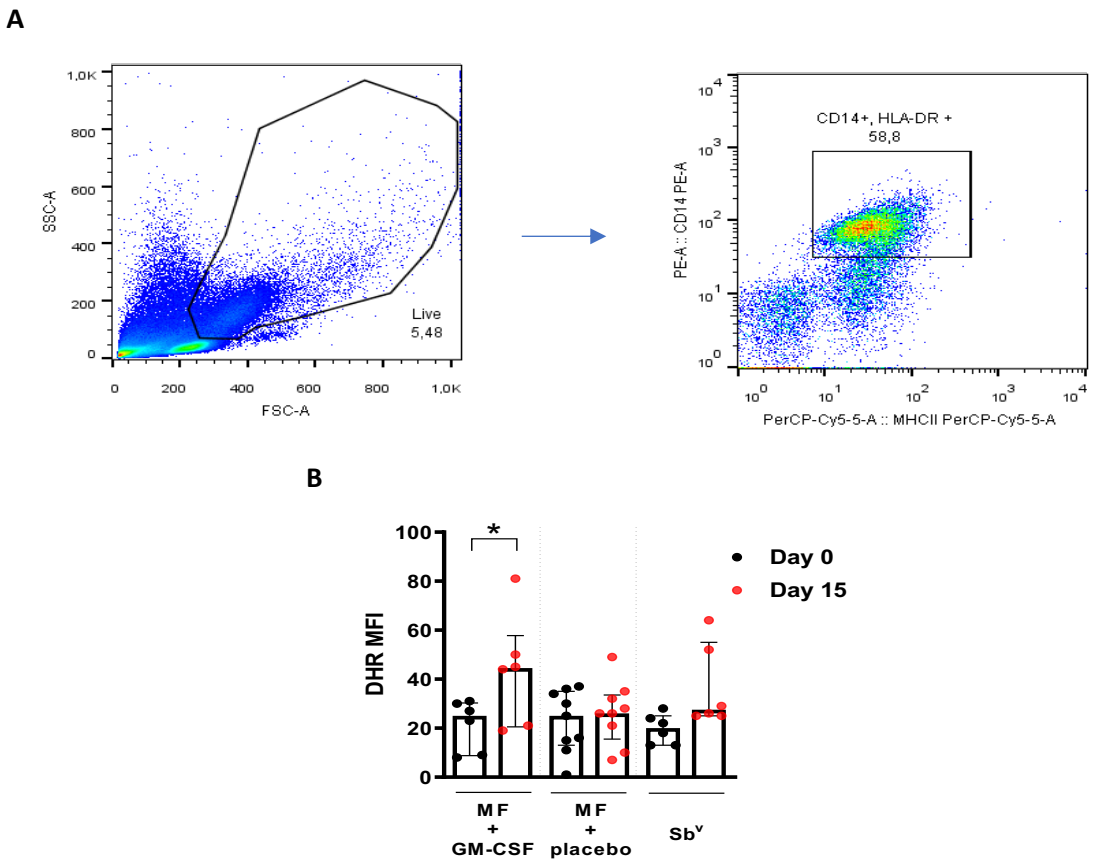

Fig 2. Miltefosine *plus* GM-CSF treatment enhance reactive oxygen species production by monocytes after *L. braziliensis* infection. Monocytes from CL patients were evaluated on day 0 and 15 of treatment with miltefosine + GM-CSF (n=6), miltefosine + placebo (n=9) and Sb^v (n=6). The cells were treated with DHR (10ng/mL – 10 min) and infected with *L. braziliensis* promastigotes for 25 minutes at a ratio of 5:1 cell. Cells were stained with anti-CD14 and anti-HLA-DR. Data were collected using flow cytometry and analyzed FLOWJO® software. (A) Representative gating strategy on $CD14^+$ and $HLA\text{-}DR^+$ expression in monocytes from one CL patient. DHR MFI was taken from $CD14^+$ $HLA\text{-}DR^+$ population. (B) The data represent the mean of fluorescence intensity (MFI) of oxidative burst production by monocytes from CL patients insert in the treatment groups. Statistical analyses were performed using the Mann-Whitney test for unpaired groups and Wilcoxon rank test for paired measurements *p<.05.

Figure 3

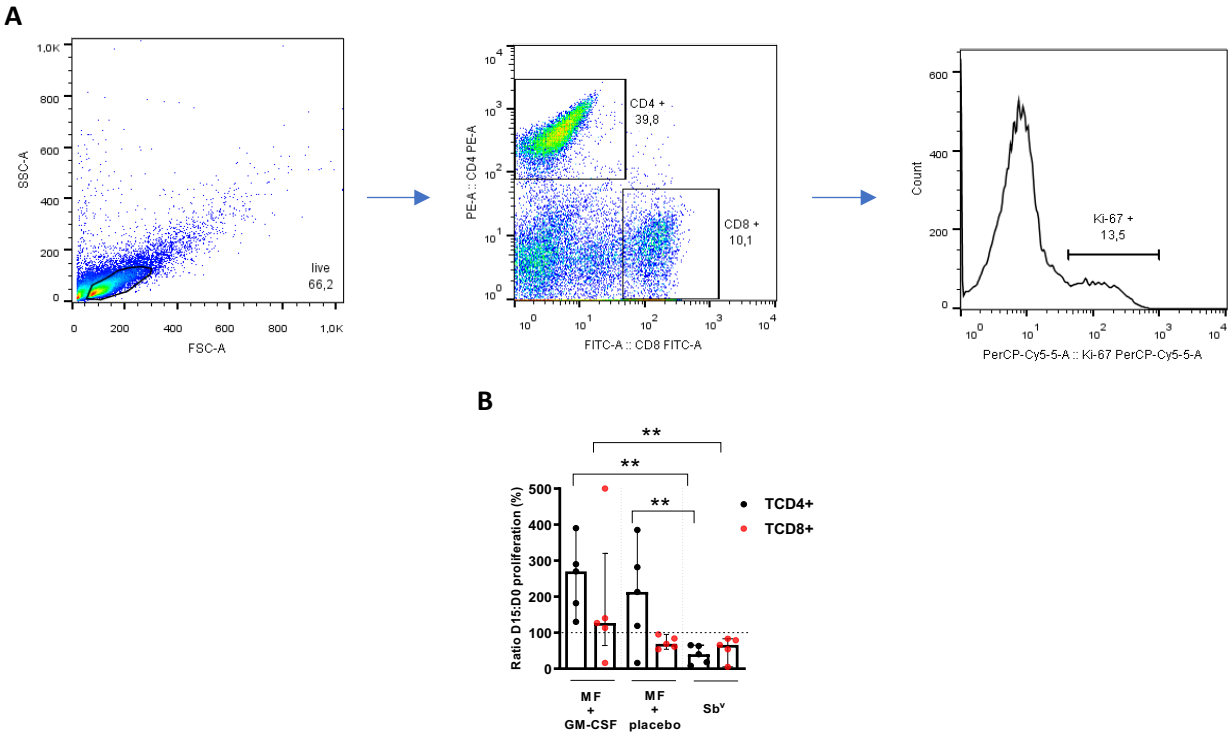

Fig 3. Miltefosine treatment inducts CD4$^+$ T cells proliferation and its association with GM-CSF enhances CD8$^+$ T cells proliferation by PBMC from CL patients. PBMC from CL patients treated with miltefosine + GM-CSF (n=5), miltefosine + placebo (n=5) and Sb^v (n=5) were cultured for 5 days in presence of SLA on day 0 and 15 of therapy. Cells were stained with anti-CD4, anti-CD8 and anti-Ki67. Data were collected using flow cytometry and analyzed FLOWJO® software. (A) Representative gating strategy on CD4$^+$, CD8$^+$ and Ki-67$^+$ expression in lymphocytes from one CL patient. (B) The data represent the ratio between the proliferation found at day 15 and day 0 of treatment from CL patients insert in the treatment groups added by 100. Statistical analyses were performed using the Mann-Whitney test for unpaired groups and Wilcoxon rank test for paired measurements *p<.05, **p<.01

Figure 4

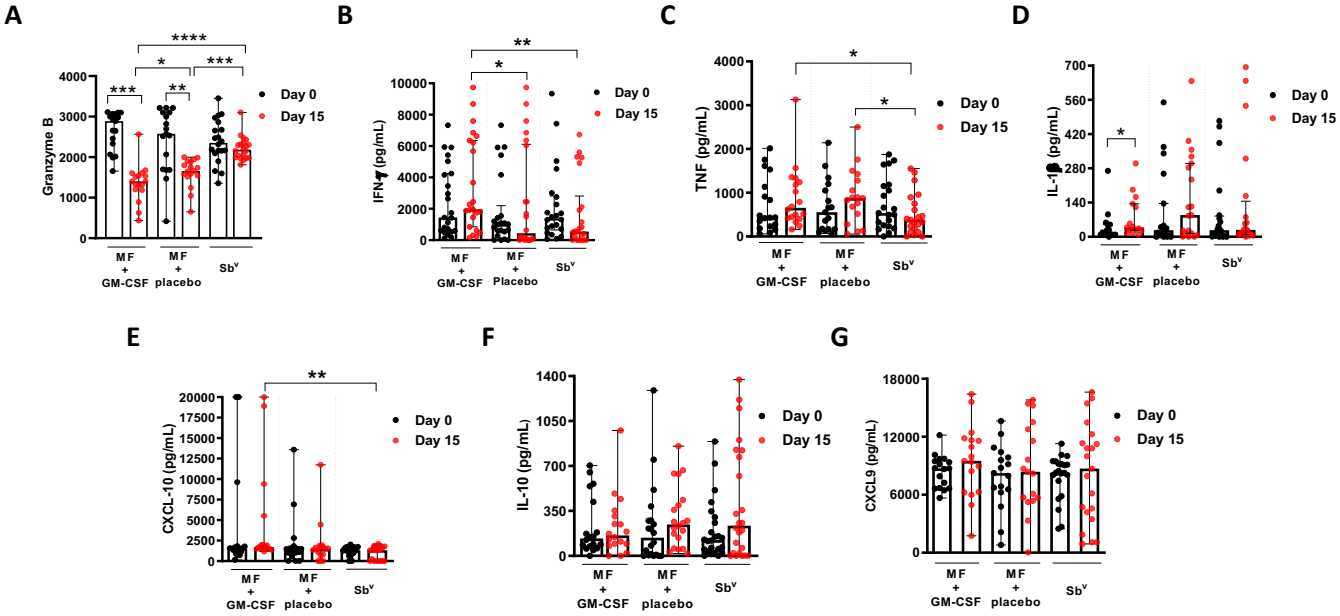

Fig 4. Cytokine production by PBMC from CL patients during therapy. PBMC from patients treated with miltefosine + GM-CSF (n=17), miltefosine + placebo (n=17) and Sb^v (n=21) were stimulated with SLA (5ug/mL) for 72 hours on day 0 and 15 of therapy. (A) Granzyme B, (B) IFN-γ, (C) TNF, (D) IL-1β, (E) CXCL-10, (F) IL-10 and (G) CXCL-9 levels were determined in culture supernatants by ELISA. Statistical analyses were performed using the Wilcoxon or Mann-Whitney rank test *p<.05, **p<.01, ***p<.001, ****p<.0001.

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